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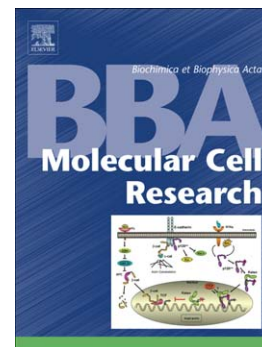
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Identification of novel peptide motifs in the serpin maspin that affect vascular smooth muscle cell function

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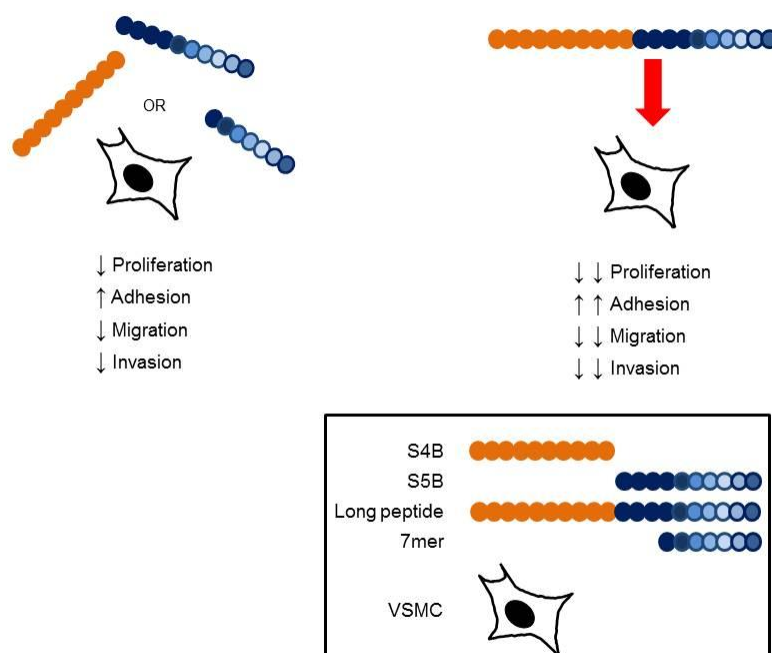
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Abstract

Maspin is a non-inhibitory member of the serpin family that affects cell behaviours related to migration and survival. We have previously shown that peptides of the isolated G α -helix (G-helix) domain of maspin show bioactivity. Migration, invasion, adhesion and proliferation of vascular smooth muscle cells (VSMC) are important processes that contribute to the build-up of atherosclerotic plaques. Here we report the use of functional assays of these behaviours to investigate whether other maspin-derived peptides impact directly on VSMC; focusing on potential anti-atherogenic properties. We designed 18 new peptides from the structural moieties of maspin above ten amino acid residues in length and considered them beside the existing G-helix peptides. Of the novel peptides screened those with the sequences of maspin strand 4 and 5 of beta sheet B (S4B and S5B) reduced VSMC migration, invasion and proliferation, as well as increasing cell adhesion. A longer peptide combining these consecutive sequences showed a potentiation of responses, and a 7-mer contained all essential elements for functionality. This is the first time that these parts of maspin have been highlighted as having key roles affecting cell function. We present evidence for a mechanism whereby S4B and S5B act through ERK1/2 and AMP-activated protein kinase (AMPK) to influence VSMC responses.

Graphic Abstract



Schematic depiction of the effect of maspin peptides on VSMC behaviours

Key words

Maspin, serpin peptide, migration, adhesion, proliferation, invasion

Abbreviations

VSMC, vascular smooth muscle cells; S4B, strand 4 of beta sheet B; S5B strand 5 of beta sheet B; RCL, reactive centre loop; AMPK, AMP-activated protein kinase; G-helix, G α -helix; S1A, strand 1 of beta sheet A; S2C, strand 2 of beta sheet C.

Introduction

The non-inhibitory serpin maspin (SerpinB5) affects cell behaviours that are consistent with its initial identification as a tumour metastasis suppressor in breast carcinoma [1]. Since then numerous studies have demonstrated that it decreases tumour growth, invasion, metastasis and angiogenesis (recently reviewed [2]). Maspin affects a range of cell types including those that do not express it. Commonly expressed by epithelial cells and lost in carcinogenesis, it has also been shown to be expressed by and influence endothelial cells [3], lymphocytes [4] and smooth muscle cells [5]. We have shown that maspin affects the functions of vascular smooth muscle cells (VSMC) that impact on the development of atherosclerosis; also referred to as atherogenesis [6-8].

VSMC migration and proliferation are important in the response to injury and the pathogenesis of vascular disease [9, 10]. VSMC surrounding normal blood vessels are differentiated which means that they do not proliferate or migrate [11]. One of the responses to vascular injury is that VSMC dedifferentiate, becoming motile and proliferative [11]. Whether migration and proliferation of VSMC are beneficial or not depends on the stage of the disease. Migration of VSMC to sites of vascular injury is involved in the early stages of atherosclerosis and in restenosis following angioplasty; the VSMC then proliferate *in situ*. The balance between proliferation and apoptosis of VSMC is important in atherosclerotic plaque development. Once an atherosclerotic plaque is established it is dependent on VSMC for stability – plaque rupture triggers thrombus formation, leading to heart attack or stroke. The VSMC functions contributing to these behaviours can be influenced by maspin which prevents migration, invasion and proliferation, while increasing adhesion – consequences that can generally be thought of as anti-atherogenic.

We have previously demonstrated that bioactive peptides from the maspin structure can influence VSMC behaviour. We found that a peptide of the G α -helix in isolation (G-helix) replicated the effect of full-length maspin in reducing the migration of a variety of cell types including VSMC, and contributed to the action of maspin on cell adhesion [8]. Subsequently other maspin derived peptides have been shown to have biological effects. Peptides from beta sheets strand 1A (S1A) and strand 2C (S2C) of maspin were reported to cause epithelial cell adhesion [12]. In support of our demonstration of the influence of the G-helix on cell migration and adhesion, this region of maspin has independently been shown to affect these functions in endothelial cells, resulting in the inhibition of angiogenesis when incorporated into supramolecular nanostructures [13].

How maspin derived peptides affect cell function is an intriguing question. It is possible that they replicate a subset of the protein-protein interactions of the whole protein. Like PAI-2 [14], maspin does not have a defined signal sequence but is found in both intra- and extra-cellular locations [8, 12, 15], with binding partners characterized in both contexts. We have been interested in how maspin in the extracellular environment can influence VSMC behaviour, since discovering that it acts without being able to directly inhibit serine protease activity [6]. We found that maspin directly bound β 1 integrins on the VSMC surface affecting adhesion and migration [7]. Maspin – integrin interactions have also been demonstrated on the surface of epithelial and endothelial cells [3, 16]. Overall these studies imply that there are both direct and indirect contributions of maspin to major cellular processes.

The cell-signalling pathways associated with maspin are not well understood, and have not been reported for maspin peptides previously. There is some evidence for an overlap with integrin-associated signalling

pathways. The interaction of maspin with small GTPases Rac-1 and Cdc42 has been reported to modulate PI3K and ERK1/2 [17] and JNK kinase and AP-1 [18] in breast carcinoma and FAK in endothelial cells [3]. FAK along with Akt has been implicated in how maspin influences apoptosis and angiogenesis in prostate cancer [19]. Multiple signals have been associated with VSMC proliferation and migration, with AMPK (AMP-activated protein kinase), ERK 1/2 and Akt commonly reported [20], however there have been no previous reports of maspin interacting with these signalling pathways in VSMC.

In this study we designed eighteen new peptides from the structural moieties of maspin, in addition to the G-helix, and functionally screened them to determine how they influenced the adhesion and proliferation of VSMC. The initial screens indicated that six peptides could alter cellular activity. We designed specific peptide controls for each of these six peptides and subsequently identified two peptides which displayed significant bioactivities. Strand 4 & 5 of maspin beta sheet B (S4B and S5B) decreased VSMC proliferation, migration, and invasion, while increasing adhesion. As the sequences for these regions are consecutive we tested a long peptide of the sequences of S4B and S5B together – which showed enhanced effects in comparison to the isolated peptides. Sequential removal of residues from either end of S5B allowed the identification of a minimal active region comprising a 7-mer. Insights into how these sequences influence VSMC function were provided by studies demonstrating changes in ERK1/2 and AMPK signalling in response to S4B and S5B.

Materials and Methods

Peptides, antibodies and materials - 19 peptides corresponding to discrete structural moieties of maspin were synthesized by Pepceuticals (Leicestershire, UK), with biotin-aHx on their N terminus and an amide on their C terminus. Peptides were prepared at 95% purity and dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10mM. Test peptides are detailed in table 1. The G-helix control peptide was used as a comparison for the initial screens [8]; additional control peptides were subsequently designed to match the charge and composition of their respective test peptides (Table 2). A long maspin peptide and matching control were synthesized; this comprised the total length of peptides S4B and S5B, which are adjacent in the maspin amino acid sequence (Table 1). Attenuated S5B peptides are detailed in Table 3. Peptides were usually used at a final concentration of 10 μ M in assays, which had previously been defined as optimal [8]. Anti- β 1 (Mab17781, 1:1000) integrin was from R&D Systems (Abingdon, UK). β 3 integrin (MAB20232, 1:1000) MAb was purchased from Millipore (Hertfordshire, UK). Antibodies to ERK1/2 (9102S, 1:1000) and ERK1/2P (9101, 1:1000) were from New England Biolabs UK (Hertfordshire, UK). Anti- AMPKa (GTX50863, 1:1000) and AMPKaP (GTX63165, 1:1000) were from Source Bioscience (Nottingham, UK). Fluorescent dye DiIC16 was from Life Technologies (Paisley, UK). WST-1 was purchased from Roche (Burgess Hill, UK). All other reagents were purchased from Sigma Aldrich.

Table 1

Peptide	Sequence
RCL	329-DGGDSIEVPGARIL
Helix A	4-LQLANSFAVDLFLKQLCEKE
Helix B	33-ICLSTSLSLAQVGA
Helix C	48-DTANEIGQVLH
Helix D	65-DIPFGFQTVTSDVNKL
Helix G	236-EDESTGLEKIEKQLN
Helix F	126-EETKGQINNSIKDLTD
Helix H	252-NSESLSQWTN
Helix I	285-KACLENLGLK
S2A	74-YSLKLIKRLYVDKS
S3A	158-KILVVNAAYFVG
S5A	317-NVIHKVCLEIT
S1B	190-TDTKPVQMMNMEA
S2B	213-NCKIHELPFQN
S3B	225-HLSMFILLPKD
S4B	354-FIYIIRHNKT
S5B	364-RNIIFFGKFC
S4B/S5B Long	354-FIYIIRHNKTRNIIFFGKFC
S2C	260-NPSTMANAKVKLSIP
S3C	181-TKECPFRLNK

Table 2

Peptide	Sequence
G Helix Control (C-Pep)	EDESTGELKILKQEN
RCL Control	EAAESIDVPAGRIL
S2A Control	YSIRILRKIYVDRS
S3A Control	KIVLLNGGYFLA
S5A Control	TLIHKLCVEIN
S4/5B Control	FIKNRHTYCG
S4B/S5B Long Control	FIKNRHTYCGFIKNRHTYCG

Table 3

Peptide	Sequence
Minus 1L	NIIFFGKFC
Minus 1R	RNIIFFGKF
Minus 2L	IIFFGKFC
Minus 2R	RNIIFFGK
Minus 3L	IFFGKFC
Minus 3R	RNIIFFG
Minus 4L	FFGKFC
Minus 4R	RNIIF
Minus 5L	FGKFC
Minus 5R	RNIIF
Minus 6L	GKFC
Minus 6R	RNI

Cell Culture - Primary aortic smooth muscle cells (referred to as VSMC), were cultured as described previously [7] in 231 medium supplemented with smooth muscle cell growth supplement containing 5% (v/v) FBS. For some experiments, VSMC were transferred into phenol red free serum-free MEM. VSMC were used between passages 3 and 8. HT29 cell line was purchased from ATCC and cultured in DMEM, supplemented with 10% (v/v) FBS, L-glutamine and penicillin/streptomycin. All cell culture reagents including extracellular matrix (ECM) components were from ThermoFisher Scientific (Paisley, UK).

Kinetic Adhesion and Proliferation Assay - To screen the 19 maspin peptides for their ability to alter adhesion and proliferation of VSMC, an xCELLigence DP real time cell analyser was used according to the manufacturer's instructions (Acea Biosciences Inc., CA, USA). Briefly VSMC were seeded onto an xCELLigence E plate at 6000 cells/well with maspin peptides or control. E plates consist of a gold microelectrode and attachment/spreading of cells is determined by measuring relative electrical impedance across the cell monolayer. Adhesion was determined by recording impedance every 5 minutes for 4 hours. Following this 4 hour period, impedance measurements were then taken every 15 minutes for up to 72 hours to determine cell proliferation as described previously [21]. In some experiments, E plates were coated with ECM components at 5µg/ml for 15 hours at 4°C and then blocked with 1% (w/v) BSA for 30 minutes at 37°C prior to the addition of VSMC.

Endpoint Adhesion Assay - Endpoint assays were used to measure cell adhesion to individual ECM components or fibrillar matrix. Wells were coated with fibrillar matrix made by HT29 cells as described previously [8] or individual ECM components as for the kinetic assays. VSMC were labelled with DilC16 fluorescent dye in phenol red free, serum free MEM for 30 minutes at 37°C, then centrifuged at 800g for 5 minutes and resuspended in fresh medium. Cells were incubated with maspin peptide for 30 minutes at 37°C, prior to plating onto the fibrillar matrix/ECM coated wells at a density of 6000/well. Cells were incubated at 37 °C for 45 minutes then washed with PBS to remove non-adherent cells. Fresh medium was added to each well and fluorescence intensity of the plate was measured at Ex544, Em590 using a FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK). In some experiments labelled VSMC were pre-treated with anti-β1 or anti-β3 integrin at 5µg/ml for 30 minutes at 37°C.

Endpoint Proliferation Assay - VSMC were plated onto 96 wells at a density of 5000 cells/well and cultured overnight. The cells were serum-starved in 0.5% (v/v) FBS containing medium for 24 hours, then cultured in complete growth medium containing maspin peptides for a further 24 hours. To quantify cell proliferation, WST-1 was added to each well and cells were incubated at 37°C for 5 hours to allow colour change to develop. Absorbance at 490nm was measured using a FLUOstar Omega plate reader.

Kinetic Migration and Invasion Assay - Migration and invasion of VSMC in response to maspin peptides was determined using an xCELLigence DP according to the manufacturer's instructions. Migration was as described previously [21]. Invasion of VSMC was investigated using CIM plates coated with collagen gel. 90% (w/v) rat tail collagen, 10x DMEM, 10mM NaOH was added to the top chamber of the CIM plate and allowed to polymerize at 37°C. VSMC were prepared as for migration assays and added to the top of the collagen gel. Impedance measurements were recorded every 15 minutes over a 24 hour period. Cell index plots were used to calculate area under curve values to give one overall measurement of cell migration/invasion for each well of the xCELLigence plate using Origin Software (Silverdale Scientific, Buckinghamshire, UK). These values were then expressed as percentage migration relative to control peptide.

Cell-based ELISA - Activation of signalling molecules ERK1/2 and AMPK in VSMC in response to maspin peptide treatment was determined by semi-quantitative cell-based ELISA. VSMC were seeded onto 96 wells at a density of 5000/well and cultured overnight. The cells were serum-starved for 24 hours, then cultured in complete growth medium containing maspin peptides for a further 24 hours. Medium was removed from the wells and cells were fixed for 20 minutes at room temperature in 4% (w/v) paraformaldehyde. Fixed cells were then washed x3 for 5 minutes with PBS +0.1% (v/v) Tween 20,

followed by incubation with 2% (v/v) hydrogen peroxide solution in PBS for 20 minutes at room temperature. Wells were blocked with 5% (w/v) BSA for 1 hour followed by incubation with primary antibody overnight at 4°C. Cells were then incubated with an HRP-conjugated secondary antibody for 1 hour at room temperature and developed with TMB substrate. The reaction was stopped with 2.5M H₂SO₄ and absorbency of the plate was measured at 450nm using FLUOstar Omega plate reader.

Statistical Analysis - Statistical analysis was carried out using Microsoft Excel and Origin software packages. Statistical analysis was performed on raw data, prior to processing to data relative to control which was used for presentation to aid understanding. Data are presented as mean±SEM. For single comparison tests, significance was judged using Student's t test. For multiple comparisons ANOVA followed by Tukey's post-test was used. A value of $p < 0.05$ was considered statistically significant.

Results

S4B and S5B are potentially important structural motifs for maspin function

A panel of 19 peptides was designed, corresponding to discrete structural motifs in the maspin molecule (Table 1). The peptides were initially screened using for their ability to affect VSMC adhesion (Figure 1) and proliferation (Figure 2). We had previously demonstrated that 10µM was the optimal concentration for the use of G-helix peptides in cell culture models [8], so used this as a starting point. The effectiveness of this concentration was verified with some peptides (supplementary figure 1).

The changes in VSMC cell adhesion (Figure 1A) and proliferation (Figure 2A) in the presence of peptides on six matrix conditions were determined. Peptides RCL (reactive centre loop), S2A, S5A, S4B and S5B significantly increased VSMC adhesion to ECM components when compared to the control peptide C-Pep used in previous studies [8] (Table 2). When specific control peptides were designed to be matched for charge and length to each of these peptides, S4B and S5B increased cell adhesion by at least 50% using an endpoint adhesion assay, when compared to a peptide-specific control (Figure 1B and Table 2). RCL, S2A and S5A showed no significant increase compared to their specific controls (supplementary Figure 2). S4B was also able to increase VSMC adhesion to fibrillar matrix produced by the HT29 cell line by $48 \pm 10\%$, whereas S5B showed no significant increase (Figure 1B). We have previously demonstrated that the ability of maspin to increase adhesion to fibrillar matrix was not dependent on the type of cell used to deposit the matrix [8].

Peptides G helix, S3A, S4B and S5B were identified from the initial screen against C-Pep as able to significantly reduce VSMC proliferation (Figure 2A). When these were further tested only S4B and S5B significantly reduced proliferation by at least 17% compared to the peptide-specific control (Figure 2B). Titration of the S4B and S5B peptides in proliferation assays demonstrate that 10µM is the optimal for these peptides (supplementary figure 1). The effect of S4B and S5B on other cell types was investigated and it would appear that there is specificity to VSMC (supplementary figure 2). G helix and S3A caused no significant reduction in proliferation compared to their specific controls when assessed by WST-1 viability assay (supplementary figure 3). This was disappointing as S3A had the most universal ability to decrease proliferation in the initial screen against the C-Pep peptide. Overall the initial high through put screen, followed by validation experiments using endpoint assays and peptide-specific controls demonstrated that the peptides in isolation could influence important VSMC functions. It also allowed the identification of structural motifs of maspin that have not previously been linked to functional importance. S4B and S5B are potentially critical for the actions of maspin as both can increase the adhesion and decrease the proliferation of VSMC.

Peptides S4B and S5B decrease migration and invasion of VSMC

The importance of the motifs S4B and S5B for the actions of maspin was further investigated by determining the effect of their corresponding peptides on the migration (Figure 3A) and invasion (Figure 3B) of VSMC. S4B and S5B peptides significantly decreased the migration of VSMC by as much as 64% when compared to specific control peptide (Figure 3A). Both peptides also reduced the invasion of VSMC through a cross-linked collagen gel by 50% compared to control peptide (Figure 3B). These data suggest that peptides S4B and S5B in isolation can alter the behaviours of VSMC also shown to be mediated by the intact maspin molecule [6].

Maspin S4B/S5B long peptide also influences VSMC behaviour

In the maspin molecule the sequences of S4B and S5B are consecutive. As each peptide individually could influence VSMC function, a long maspin peptide consisting of the entire sequence of both of these motifs was synthesized (Table 1) in addition to a long peptide control (Table 2). The long maspin peptide was then tested for its ability to alter behaviour of VSMC. The long peptide increased VSMC adhesion to ECM components and fibrillar matrix compared to long peptide control by between 35-125% (Figure 4A). In particular adhesion to vitronectin was dramatically increased, demonstrating an increase in adhesion of $125 \pm 31\%$. The long peptide also significantly decreased proliferation by $27 \pm 18\%$ (Figure 4B) and migration by $60 \pm 4\%$ of VSMC (Figure 4C) compared to long peptide control. So the long peptide potentiated the effects observed for S4B and S5B individually.

Maspin peptides affect cell signalling pathways involved in cell migration

Having demonstrated that the peptides S4B and S5B alone or when synthesized in combination could influence the behaviour of VSMC, we sought to understand the mechanism of action. We investigated the effect of maspin peptides on the activation state of signalling molecules AMPK and ERK1/2, which are known to be involved in cell migration [22, 23]. Using cell-based ELISA we looked at the effect of maspin peptides S4B and S5B on ERK1/2 phosphorylation (Figure 5A) and the effect of the long peptide (Figure 5B). All three peptides significantly decreased ERK1/2 phosphorylation by up to 32% in comparison to corresponding peptide controls; the total ERK1/2 detected was unchanged.

In contrast, AMPK phosphorylation was increased by up to 30% in response to S4B and S5B (Figure 5C) and long peptide (Figure 5D). AMPK has been previously shown to decrease migration of VSMC [23, 24]. To test whether the decrease in migration mediated by maspin peptides required AMPK, VSMC were treated with the AMPK inhibitor dorsomorphin and migration in response to the long peptide was determined. After treatment with dorsomorphin, the inhibitory effect of the long peptide on VSMC migration was significantly attenuated compared to migration of VSMC treated with the long peptide alone (Figure 5E). Dorsomorphin had no significant effect on VSMC migration in response to the long peptide control, suggesting that the long peptide and therefore maspin may act through AMPK to reduce cell migration.

Adhesion by peptide S4B may involve $\beta 1$ integrin

We have shown previously that maspin reduces migration of VSMC through interactions with $\beta 1$ integrin [7]. To test whether the increase in cell adhesion observed with maspin peptides also involved $\beta 1$ integrin, VSMC were pre-incubated with blocking antibodies to either $\beta 1$ or $\beta 3$ integrin and adhesion to fibronectin in response to maspin peptides determined. Treatment with either antibody resulted in a significant reduction in S4B mediated cell adhesion compared to cells treated with S4B alone (Figure 5F). The anti- $\beta 1$ reduced the adhesion of VSMC + S4B by 69%, in comparison to the anti- $\beta 3$ which reduced adhesion by 20%. Only cells treated with $\beta 1$ MAb adhered less than cells treated with S4/5B specific control peptide (reduction of $54 \pm 12\%$). Neither antibody had any significant effect on S5B peptide-mediated adhesion (Jenkinson & Bass, unpublished observations).

Identification of minimal active region

In addition to considering longer peptides we investigated the effect of reducing the size of S5B to identify a minimal active region. Peptides were made by sequentially removing amino acid residues from each end of S5B. The ability of the shortened peptides to alter adhesion (Figure 6A) and migration (Figure 6B) was investigated. The ability of S5B to increase adhesion was maintained until four amino acid residues had been removed from the peptide. This indicated that a 7-mer peptide (sequence IFFGKFC) contained the essential elements for increasing adhesion. This region was also able to reduce migration, when removal of another residue abolished the effect.

Discussion

Here we demonstrate the identification of previously unreported peptides from the sequence of maspin that can influence VSMC proliferation, adhesion and migration. It is an evolution of our previous study showing that the G-helix of maspin plays an essential role in the effects of the serpin on the migration of a variety of cell types including VSMC [8]. This has been confirmed subsequently by others who reported effects of the G-helix of maspin on the migration and adhesion of endothelial cells and possible application as anti-angiogenic therapy [13]. The potential for the use of peptides from discrete structural motifs of serpins as therapeutic agents is of broader interest than those from maspin. A recent study demonstrated the utility of RCL peptides from serpin family members Serp-1 and NSP in the reduction of atherosclerotic plaque formation [25].

In this study the sequences of all 25 structural domains of maspin were considered and those consisting of at least ten amino acid residues synthesized (Table 1). Exceptions to this were peptides of S1B and S2C, which were slightly extended to encompass sequence areas previously reported to be involved in cell adhesion [12]. Initial high-throughput screens were performed relative to the G-helix control peptide, and then specific controls designed for those determined to be of interest. Representative time points are shown in this paper, but the effect of peptides was often maintained throughout the time scale of assays (Supplementary figure 4).

Unexpectedly we found that most of the peptides had at least some significant effect on cell proliferation and adhesion. Adhesion was increased by most peptides, with a peptide dependent variation in ECM specificity. Only the α -helix I had no effect on VSMC adhesion. Longer term, when assessing proliferation fewer of the peptides affected VSMC function; here we concentrated on those decreasing proliferation. It is worth noting that ten of the peptides could increase proliferation, again in a fashion dependent on which ECM component the cells were grown on; providing possible targets for exploration as maspin antagonists in future.

In line with previous findings [12] S1B and S2C had some effect on cell adhesion, but not as consistently or significantly as those observed with peptides from regions RCL, S2A, S5A, S4B and S5B. Endpoint adhesion assays were used to verify the results and investigate whether the peptides showed a significant difference to control peptides individually matched for charge and size. This was important to rule out non-specific effects. S5A and S4B significantly increased adhesion in comparison to their matched control; RCL, S2A and S5A did not. Similarly G helix, S3A, S4B and S5B were investigated in endpoint proliferation assays against specific controls; again only S5B and S4B caused comparative significant decreases. This is the first time that the contributions of these regions to the functionality of maspin (or any serpin) have been directly demonstrated.

There was a mechanistic difference in how S4B and S5B influenced the adhesion of VSMC as although both increased it by over 50% on isolated ECM components, there was no enhancement of VSMC adhesion to fibrillar matrix in the presence of S5B. The reason for this remains undetermined, but is likely related to differences in integrin engagement, as only S4B enhanced adhesion could be reduced by integrin function blocking antibodies. Conversely both peptides decreased VSMC proliferation by at least 20% indicating a similar affect.

We went on to look at whether S4B and S5B peptides were sufficient for the control of other cell functions archetypally ascribed to full length maspin. Both peptides inhibited VSMC migration and invasion in comparison to their matched control; migration by >40% and invasion by approximately 50%. These are similar to the effects of full length maspin previously reported [6, 26], so allows the probability that the peptides are working through a similar mechanism as the full length maspin. The similarity of the magnitude of responses to both peptides again allows us to speculate that both are working through the same mechanism.

It would appear that the effect of S4B and S5B are specific to VSMC as they did not inhibit the growth of breast or prostate cancer cell lines. The effect of the peptides on other functions of these cells has not yet been determined.

A long peptide encompassing the sequences of S4B and S5B together showed a slight enhancement of the functional effects demonstrated for the individual S4B and S5B peptides. Notable amongst this was an increase in cellular adhesion to vitronectin by 125%. Stepwise removal of residues from either end of S5B allowed the demonstration of a minimal region with the sequence IFFGKFC, which could influence adhesion and migration – removal of further residues abolished the effects.

We demonstrate here that peptides of the S4B and S5B motifs of maspin affect VSMC function in ways that could have a positive impact on the initial development of atherosclerotic plaques, or on restenosis after angioplasty; these peptides can be thought of as anti-atherogenic. The ability of S4B and S5B to decrease VSMC proliferation, migration and invasion, while increasing adhesion imply that they would be able to reverse VSMC behaviours required for the formation of atherosclerotic plaques.

The sequences of S4B and S5B are located near the C-terminus of maspin. They are next to each other in both the sequence and the three dimensional structure, where they are found in the hydrophobic core [27]. Although not predicted to be on the surface of the molecule these regions are in a position adjacent to a solvent accessible cleft, which could provide an insight into the functionality of this region. The sequences of S4B and S5B are highly conserved and unique to maspin orthologues (SIB BLAST service), implying that this region has an important role in full length maspin.

To provide insights into the mechanism of action cell signalling pathways were studied. We found evidence that S4B and S5B peptides individually and together in the long peptide work through ERK1/2 and AMPK. All three peptides decreased ERK1/2 activation and increased the levels of active AMPK; in addition an AMPK inhibitor partially attenuated the effects of the long peptide on VSMC migration. There are numerous studies linking the activation of AMPK to decreases in VSMC proliferation in both cell and animal models [20, 24, 28, 29]. This allows a potential explanation for how the downstream effects of the peptides are mediated. Suppression of ERK1/2 activation has been linked to a decrease in VSMC migration, potentially via an increase in on AMPK activation [20]. The activation of AMPK has been reported to decrease the migration of colon cancer cells by a β 1 integrin dependent mechanism [30], providing a link to the influence of S4B and S5B on adhesion. It has been suggested that AMPK activation is anti-atherogenic [24] – providing us with an explanation for these effects of S4B and S5B. The relationship between ERK1/2, AMPK and VSMC migration allows us to propose that maspin controls cell migration by increasing AMPK activation and decreasing ERK1/2 activation.

Although the direct influence of maspin on the plasminogen activation system has long been discounted [6], reports of a link between the two persist [12, 31, 32]. The ability of the peptides to interfere with plasminogen activation, due to exogenous urokinase plasminogen activator (uPA) binding to its cell surface receptor (uPAR) was investigated using well established methods [6]. As S4B and S5B were not able to alter plasminogen activation, this was not an explanation for how the peptides influence cellular function (Bass, unpublished observations). Peptides from other regions of maspin did effect plasminogen activation by up to 40% – S2A and S5A significantly enhanced it, while peptides of helices C, D and F significantly decreased it (Bass, unpublished observations).

How the S4B/S5B peptides interacted with the VSMC to induce these effects was not directly addressed. The short time required for their influence to be seen indicates a dynamic regulation of cellular processes, similar to prior observations with full length maspin [6]. The peptides could either have been taken up into cells as recently demonstrated for full length maspin [33] or acting through cell surface receptors as discussed. Either of these mechanisms could account for the effects on downstream signalling processes demonstrated.

The role of integrins in the action of S4B and S5B was investigated, as we and others have previously demonstrated the role of $\beta 1$ integrins in maspin function [3, 6, 12, 16]. Although the enhancement of cell adhesion by S4B was reduced by a $\beta 1$ blocking antibody, neither peptide interacted directly with Fc- $\alpha 5\beta 1$ protein as demonstrated for full length maspin (as described [7]. Jenkinson & Bass, unpublished observations). The anti- $\beta 1$ did not affect S5B enhanced adhesion (Jenkinson & Bass, unpublished observations) – furthering support for the two peptides influencing cell adhesion in different ways.

Peptides are widely under investigation for their beneficial effects which can be exploited therapeutically; this includes those derived from serpin structures. Peptides targeting the angiogenic growth factor systems and integrins have been found to be useful as angiogenesis targeting anti-cancer and heart disease therapies (Reviewed [34]). Indeed, nanostructures incorporating the G-helix of maspin have been shown to confer anti-angiogenic effects [13]. Peptides from pigment epithelial derived factor (PEDF; SERPINF1) have been found to mediate neurotrophic properties [35], anti-angiogenic properties [36], retinal cell survival [37] and prevent liver fibrosis through hepatic stellate cell activation [38]. The peptides from the S4B and S5B of maspin demonstrated here to influence VSMC behaviours in a way that would inhibit the build-up of atherosclerotic plaques implies a potential therapeutic use.

This is the first report of the anti-atherogenic nature of two novel bioactive maspin peptides. In future it will be of benefit to determine the impact of S4B and S5B peptides on atherosclerotic plaque build-up *in vivo*. This would be further informed by generating clones expressing maspin mutated in the S4B or S5B regions for transfection into a suitable cell model, to directly determine the role of this region in the full length molecule. Other regions of maspin are also likely to be of interest, including S3C which was demonstrated to be associated with decreased risk of developing oesophageal squamous cell carcinoma if mutated by a single nucleotide polymorphism [39]. This will be pursued by studies with further individually matched control peptides.

Acknowledgements

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Table Legends

Table 1: Sequences of maspin derived peptides

Peptides were designed from the discrete structural moieties of maspin (sequences less than 10 residues were excluded). The starting position of each peptide in the maspin sequence is indicated. Nomenclature used such that S2A is strand 2 of beta sheet A.

Table 2: Sequences of control peptides

Controls were matched for size and charge to the corresponding maspin peptide.

Table 3: Sequences of attenuated peptides

Amino acid residues were sequentially removed from each end of S5B.

Figure Legends

Figure 1: Adhesion of VSMC to extracellular matrix in response to maspin peptides

Maspin peptides were screened for their ability to alter VSMC adhesion; a final concentration of 10 μ M was used for all peptides. (A) Heat map generated from xCELLigence data showing percentage increase or decrease (relative to C-Pep control peptide) in cell adhesion to ECM components in the presence of maspin peptides. CNI: collagen I, CNIV: collagen IV, FN: fibronectin, LN: laminin, VN: vitronectin. (B) VSMC adhesion to fibronectin (open bars) or fibrillar matrix (closed bars) in the presence of peptide S4B or S5B. Adhesion is expressed as percentage increase relative to specific control peptide. Statistically significant differences are presented as assessed by ANOVA followed by Tukey's post-test. Panel (B) is combined data from three separate experiments (* p <0.05 judged using Student's t test). Data presented as mean \pm SEM.

Figure 2: Proliferation of VSMC on extracellular matrix in response to maspin peptides

Maspin peptides were screened for their ability to alter VSMC proliferation; a final concentration of 10 μ M was used for all peptides. (A) Heat map generated from xCELLigence data showing percentage increase or decrease in cell proliferation on various ECM components in the presence of maspin peptides after 24 hours. CNI: collagen I, CNIV: collagen IV, FN: fibronectin, LN: laminin, VN: vitronectin. Proliferation is expressed as percentage change relative to C-Pep control peptide. Statistically significant differences are presented as assessed by ANOVA followed by Tukey's post-test. (B) Proliferation in the presence of peptide S4B or S5B after 24 hours, compared to S4/S5B control peptide as percentage relative to control. Panel (B) is combined data from three separate experiments (* p <0.05 judged using Student's t test). Data presented as mean \pm SEM.

Figure 3: Migration and invasion of VSMC after treatment with maspin peptides S4B and S5B

The effect of peptides S4B and S5B on VSMC migration (A) and invasion through rat tail collagen gel (B) was determined. Data expressed as a percentage of specific control peptide and are combined data from three separate experiments (p <0.05 judged using Student's t test). Peptides were used at 10 μ M. Data presented as mean \pm SEM.

Figure 4: The effect of maspin long peptide on VSMC behaviour

VSMC adhesion, proliferation and migration in response to the maspin long peptide were investigated. (A) Adhesion of VSMC to ECM components in the presence of maspin long peptide. LP: long peptide, FN: Fibronectin, VN: vitronectin, CNI: collagen I, CNIV, collagen IV, FM: fibrillar matrix. (B) Proliferation of VSMC in response to long maspin peptide over 24 hours. (C) Migration of VSMC in the presence of long maspin peptide. All data are combined from three separate experiments (* p <0.05 judged

using Student's t test) and expressed as percentage relative to long peptide control. Peptides were used at 10 μ M. Data presented as mean \pm SEM.

Figure 5: The effects of maspin peptides on VSMC are mediated by the engagement of ERK1/2, AMPK and integrins

(A) Expression of activated and total ERK1/2 after treatment with S4B and S5B peptides for 24 hours. Results shown as relative expression compared with S4/5B control peptide. (B) Expression of activated and total ERK1/2 after treatment with maspin long peptide. Data shown as relative expression compared with long peptide control. (C) Expression of activated and total AMPK after treatment with S4B and S5B peptides for 24 hours. Results shown as relative expression compared with S4/5B control peptide. (D) Expression of activated and total AMPK after treatment with maspin long peptide for 24 hours. Results shown as relative expression compared with maspin long peptide control. (E) Migration of VSMC in response to maspin long peptide after pre-treatment with AMPK inhibitor dorsomorphin or DMSO carrier. Migration is expressed as percentage decrease relative to control peptide + DMSO. (F) Adhesion to fibronectin in response to S4B peptide after pre-treatment with function-blocking β 1 integrin MAb or β 3 integrin MAb. Adhesion is expressed as percentage increase or decrease relative to S4/5B control peptide. All data are combined from three separate experiments (* p <0.05 judged using Student's t test). Peptides were used at 10 μ M. Data presented as mean \pm SEM.

Figure 6: Identification of the minimal active region of S5B

VSMC adhesion to fibronectin (A) and migration (B) in the presence of 10 μ M of attenuated peptides was determined. Data expressed as percentage increase relative to control peptide. Data from three separate experiments (* p <0.05 judged using Student's t test). Data presented as mean \pm SEM.

Supplementary Figure 1: The effect of titrating peptides on VSMC proliferation

VSMC proliferation was determined in the presence of titrated peptides S4B (open bars), S5B (closed bars) and LP (hatched bars). Combined data from three separate experiments (* p <0.05 judged using Student's t test). Data presented as mean \pm SEM.

Supplementary Figure 2: S4B and S5B do not affect the proliferation of breast or prostate cancer cell lines

The effect of peptides on cancer cell lines MDA-MB-231 (breast), PC3 (prostate) and DU145 (prostate) was determined by WST-1 assay after 48 hour incubations in 10 μ M of S4B (open bars), S5B (closed bars) or S4/5B control (hatched bars). No significant changes were observed in proliferation compared to the control peptide. All data are combined from three separate experiments. Data presented as mean \pm SEM.

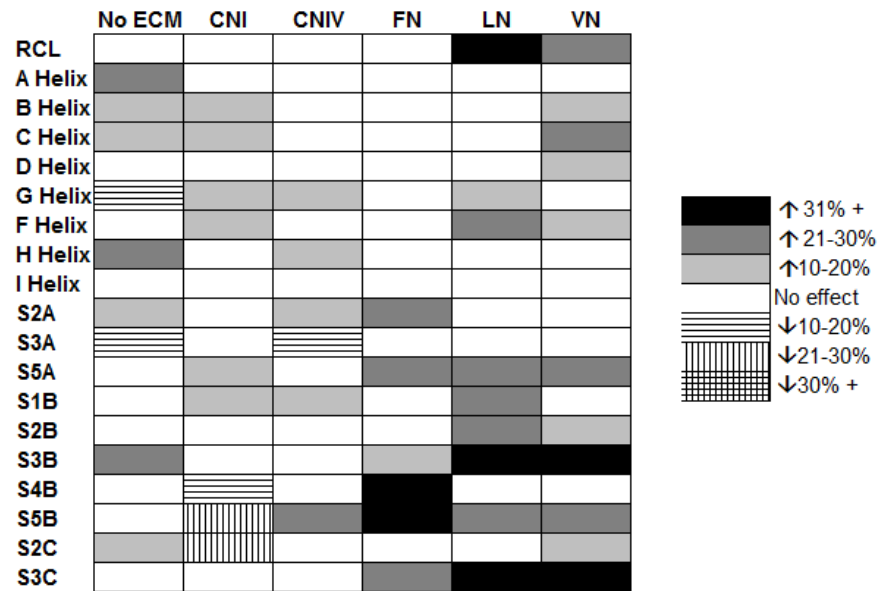
Supplementary Figure 3: Adhesion and proliferation of VSMC in response to maspin peptides

Peptides were selected for further investigation based on findings from initial screening data generated using xCELLigence DP real time cell analyser. Individual control peptides were also designed in order to test the specificity of the selected peptides to alter VSMC adhesion and proliferation. (A) Percentage adhesion of VSMC to fibronectin compared to DMSO only control. No significant change in adhesion with peptides S2A, S5A or RCL compared to their respective controls. (B) Percentage adhesion of VSMC to fibrillar matrix compared to DMSO only control. No significant increase in adhesion with peptides S2A, S5A or RCL compared to their respective controls. (C) Percentage proliferation of VSMC in the presence of G helix and S3A peptides compared to DMSO only control. No significant change in proliferation compared to control peptides. All data are combined from three separate experiments. Data presented as mean \pm SEM. Peptides were used at 10 μ M.

Supplementary figure 4: Dynamic effect of peptides on the adhesion, proliferation and migration of VSMC

xCELLigence plots showing VSMC adhesion to fibronectin over 4 hours (A), proliferation over 24 hours (B) and migration of VSMC over 24 hours (C). In all cases S4B (solid black line), S5B (dashed gray line) or control peptide (solid gray line). These are example plots, experiments were performed on at least 3 separate occasions. Peptides were used at 10 μ M.

A



B

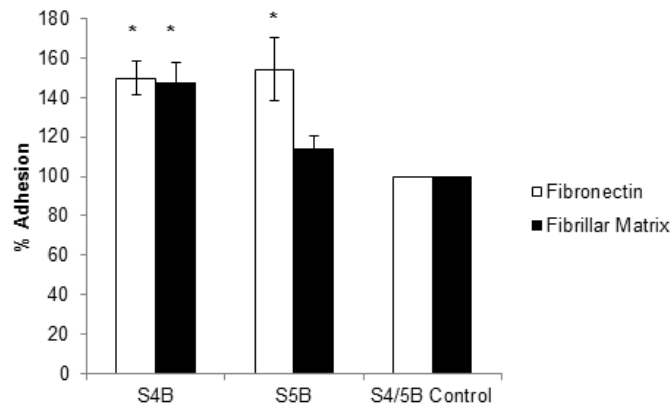
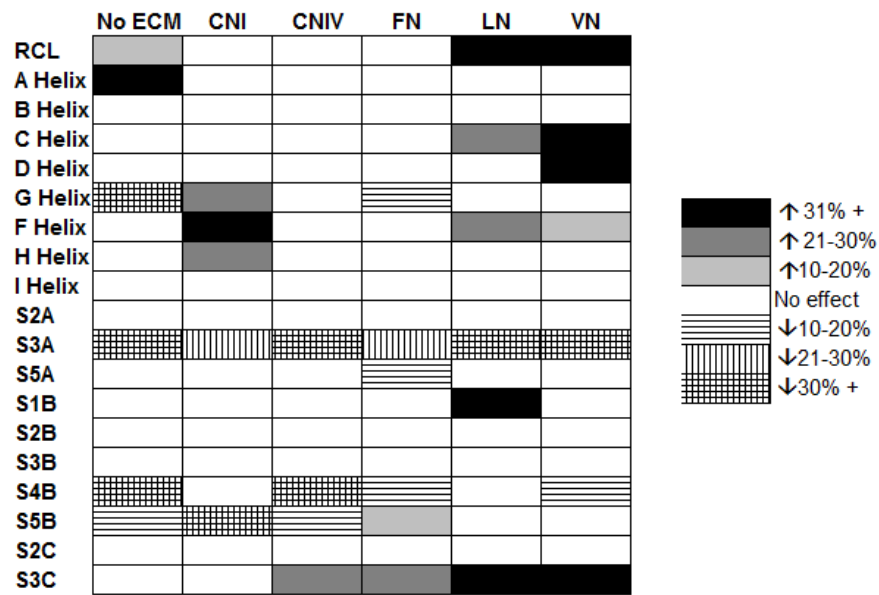


Fig. 1

A



B

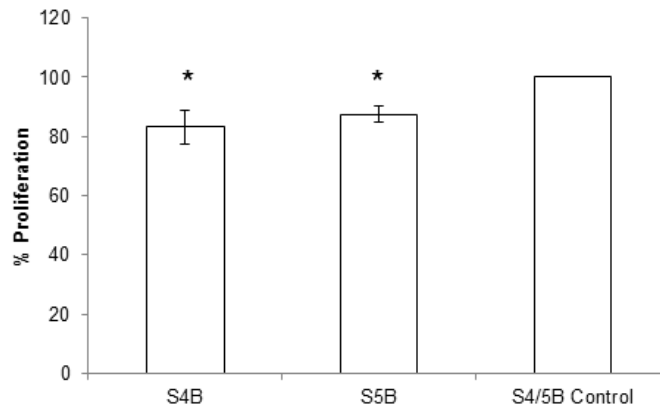


Fig. 2

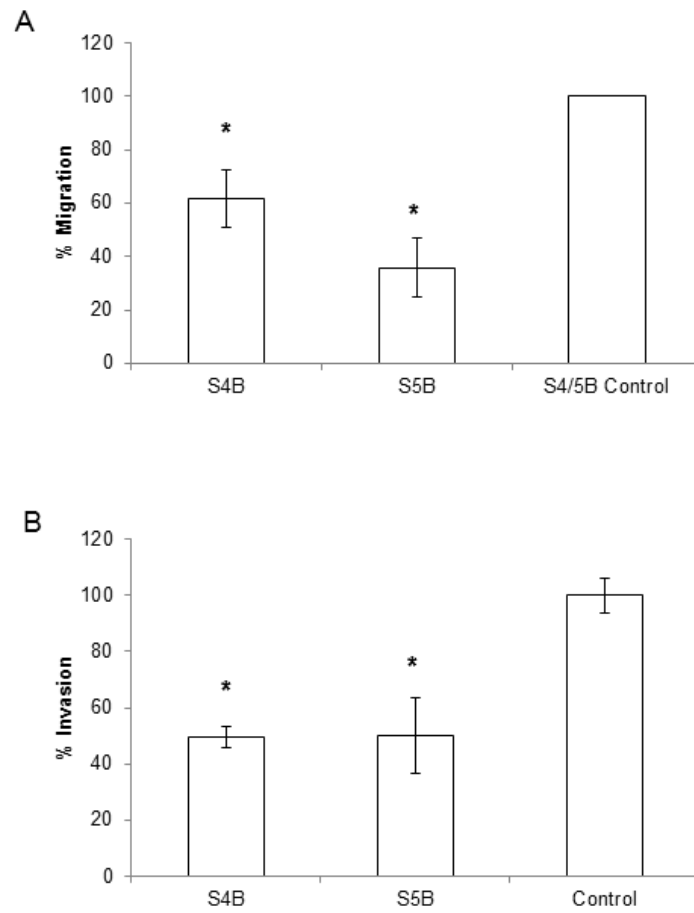


Fig. 3

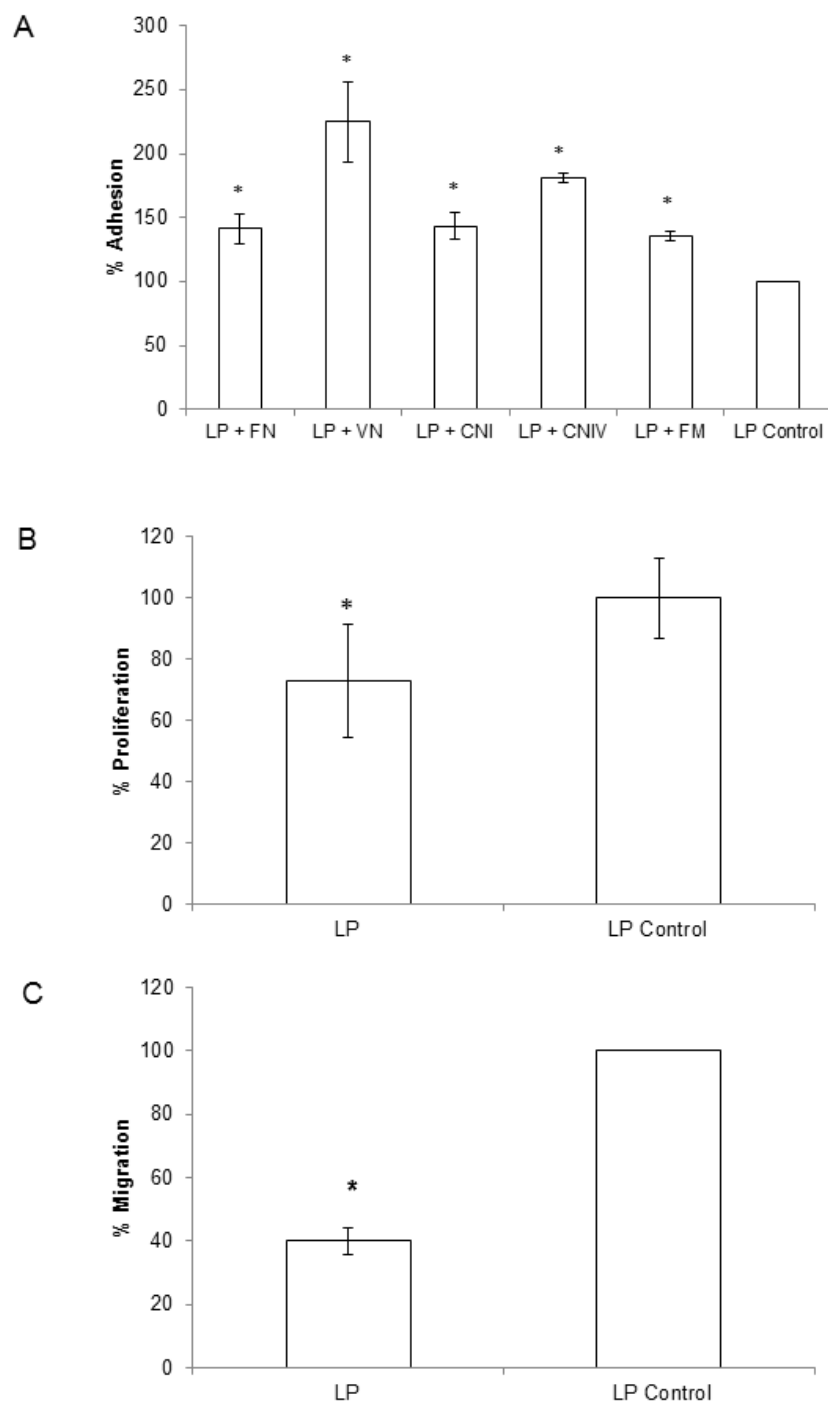


Fig. 4

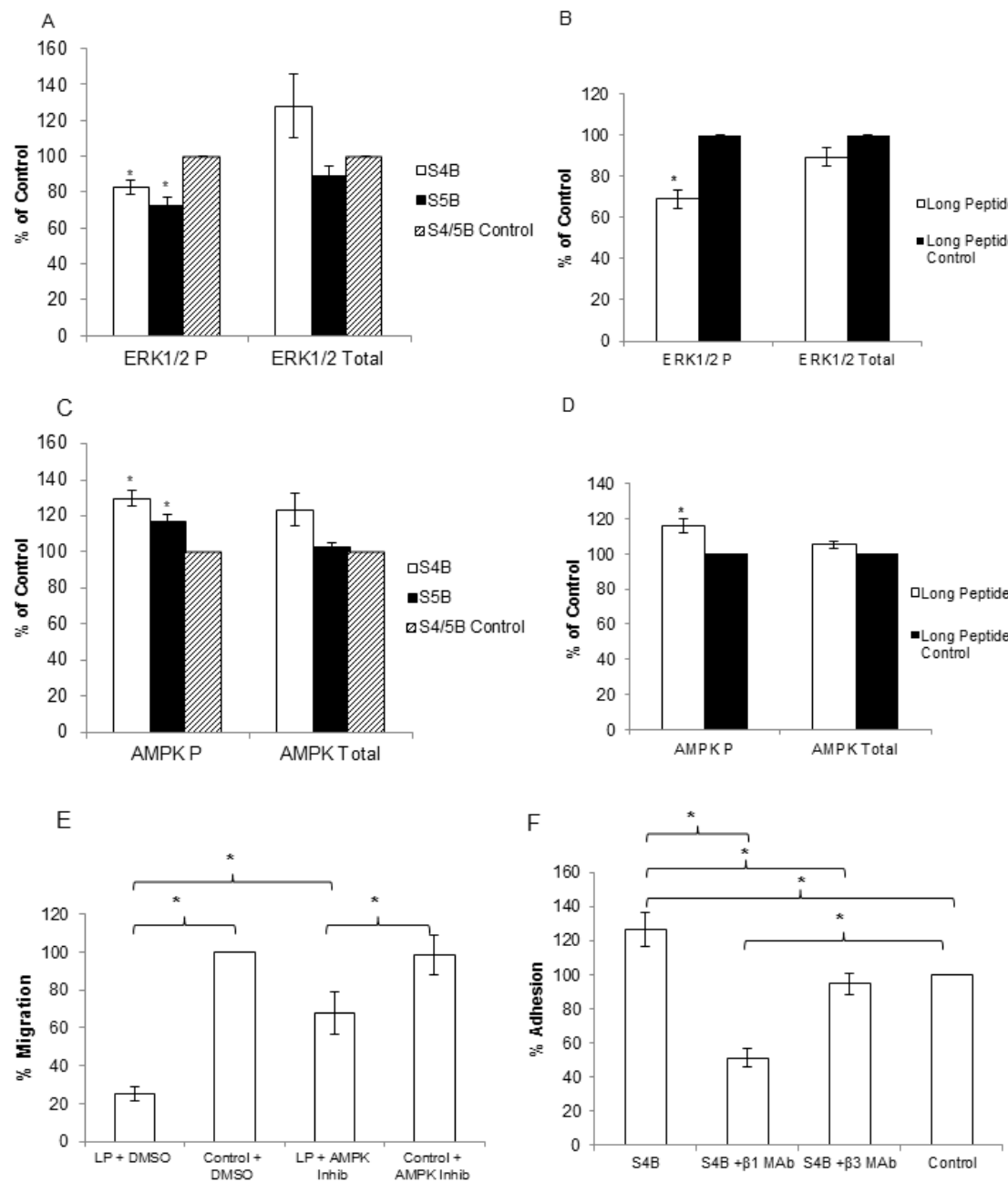


Fig. 5

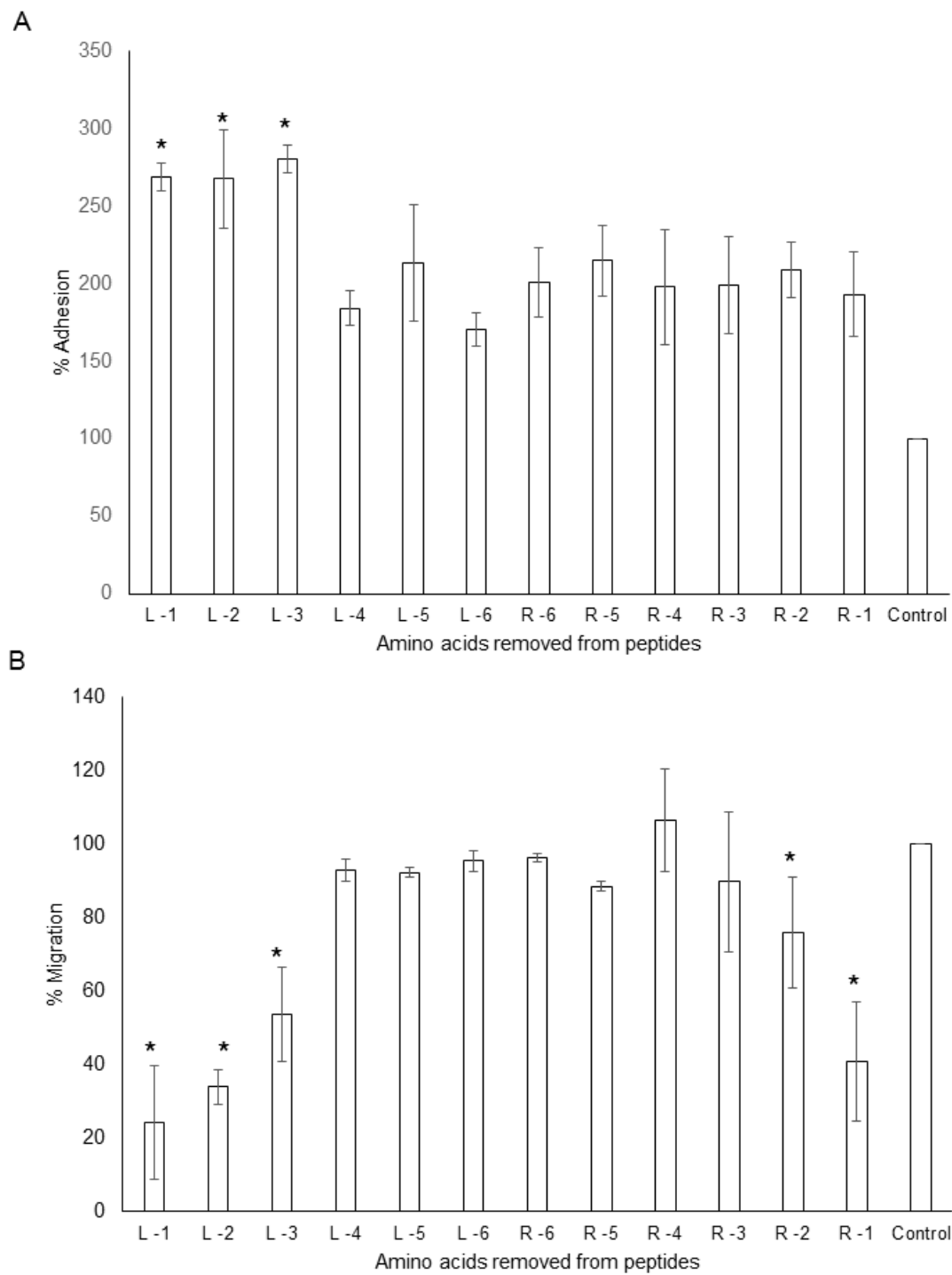


Fig. 6

Conflict of interest statement

The authors declare no conflict of interest.

ACCEPTED MANUSCRIPT

Highlights

- Peptides of S4B and S5B of maspin have anti-atherogenic actions on cultured human VSMC
- S4B and S5B peptides increased adhesion of VSMC to fibrillar and isolated extracellular matrix
- S4B and S5B peptides decreased proliferation, migration and invasion of VSMC
- A 7-mer was identified as a minimal active region
- Peptides act through decreased ERK1/2 activation and increased levels of active AMPK